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(57) Abstract

In accordance with the present invention there is provided a method for killing cancer cells expressing an activated *ras* oncogene by contacting the *ras*-activated cancer cells *in vivo* or *in vitro* with a cytotoxically-effective amount of a heterotypic antisense oligonucleotide or combination of antisense oligonucleotides, or pharmaceutically-effective analogs thereof, which have base sequences complementary to a sequence of the DNA or transcribed messenger RNA of a *raf* gene also present in the same cancer cells. The present invention also provides novel methods for treating an individual who has *ras*-activated cancer. This treatment involves the use of heterotypic antisense oligonucleotide therapies, in which a cytotoxically-effective amount of a preparation containing an anti-*raf* antisense oligonucleotide, or combination of selected anti-*raf* antisense oligonucleotides, or one or more pharmaceutically-effective analogs thereof, is administered as specific drug therapy of cancers expressing an activated *ras* oncogene.

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NOVEL METHODS AND COMPOSITIONS FOR THE TREATMENT OF RAS-ACTIVATED CANCER WITH HETEROTYPIC ANTI-RAF ANTISENSE OLIGONUCLEOTIDES

BACKGROUND OF THE INVENTION

The present invention relates to novel methods and compositions for killing cancer causing cells expressing an activated ras oncogene, and for the treatment of associated mammalian cancers. More particularly, the present invention relates to the novel use of heterotypic oligodeoxyribonucleotides which are complementary and antisense to a raf target gene sequence contained in the cells of ras-activated cancers, and which can be used as active ingredients in new anticancer therapeutic compositions.

By the term "heterotypic antisense oligonucleotide", as used herein, is meant a therapeutic antisense oligonucleotide which is specifically targeted to and binds a complementary nucleotide sequence of DNA or transcribed messenger RNA of a gene which is completely different from the cancer-causing oncogene which is known to be actively expressed in the cancer cells being treated.

In the following discussion, a number of citations from professional journals are included for the convenience of the reader. While these citations more fully describe the state of the art to which the present invention pertains, the

inclusion of these citations is not intended to be an admission that any of the cited publications represent prior art with respect to the present invention.

Cancer is a one of the most devastating and dreaded of human diseases. Much of the horror engendered by this disease derives not only from the severe debilitation often associated with its advanced stages, but also from the pain and disfigurement which frequently accompanies its clinical management. This is because much of the current therapeutic modalities (surgery, radiation treatments, intensive chemotherapy) are not able to specifically and with a high degree of accuracy kill only cancer cells; rather, by their very nature, their actions are so broad that they also kill healthy, non-cancerous bystander cells.

It would be extremely desireable to have a cancer therapy that would find and kill the cancer cells only. It is a principle object of the present invention to provide such a novel therapeutic modality for use against cancers which express a <u>ras</u> oncogene, by using a highly unique heterotypic antisense therapy directed against an apparently totally unrelated cellular gene (a <u>raf</u> gene).

To better understand the subject invention, it may be helpful to review some of the conceptual background related to

antisense oligonucleotides, oncogenes, and, in particular, ras oncogenes and raf genes.

Antisense oligonucleotide probes

Synthetic strings of DNA nucleotide bases which are complementary to the "sense" (information bearing) strand of nucleic acids have become widely recognized in recent years for their ability to inhibit the expression of specific genes (Cohen JS [editor], Oligodeoxyribonucleotides: Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL, 1989). "Antisense" oligonucleotides are single-stranded nucleic acids which, by hybridizing either to the complementary DNA nucleotide sequence in a target gene, or, more commonly, to the messenger RNA (mRNA) transcribed from that gene, are able to completely abrogate the function of the targeted gene. Because antisense oligonucleotides target RNA or DNA rather than proteins, they are drugs that can be orders of magnitude more selective than traditional drugs, a factor which should very significantly reduce problems of unwanted side effects.

The current thinking in antisense oligonucleotide therapy is to utilize homologous DNA-based oligonucleotides as therapeutic agents; i.e., as agents whose nucleotide base sequence is complementary to all or part of the nucleotide sequence of the cancer gene believed to be responsible for causing the disease. For example, in cancer cells known to

contain "activated ras oncogenes" (i.e., turned-on cancer-causing genes identified by the scientific acronym "ras" because they were discovered in viruses that cause rat sarcomas), it would be reasonable and expected to use an anti-ras antisense oligonucleotide to treat the ras-activated cancer cells; the objective being that the anti-ras antisense oligonucleotide would bind to and block the ras oncogene, thereby shutting off the transforming activity of the ras oncogene and returning the cancer cells to their normal, healthy state.

In stark contrast to current theory, the dramatic discovery of the present invention is that a heterotypic antisense oligonucleotide directed to a target gene completely unrelated to a ras oncogene can actually kill cancer cells which contain an activated ras oncogene.

The "ras" family of oncogenes.

DNA samples isolated from many long-term cultured human tumor cell lines, as well as from freshly-isolated human tumor cells, have been tested for the presence of mutated genetic sequences from known cancer-causing viruses that might be linked to the malignant transformation process. Specific viral-related genetic sequences identified in this process have been called "oncogenes." Surprisingly, many (although not all) of the mutated viral-related gene sequences identified as being responsible for the transformed state of

the cancer cell were found to be identical with (or related to) genes normally found in many, if not all, healthy cells. These normal genes (which, when mutated, can transform a healthy cell into a cancerous one) are now being called "proto-oncogenes." Unexpectedly, the normal proto-oncogenes identified in most tumor cell lines have been found to be related to the ras oncogene family (Cooper GM, Science 217: 801-806, 1982). It is extimated that between 25 and 50% of all cancers are the result of a mutation event in the genes of the ras family.

As a result of these observations, ras genes have become one of the most interesting and intensely studied oncogene families in human malignancies (Barbacid M, Ann. Rev. Biochem. 56: 779-827, 1987). While the function of ras oncogenes in facilitating the development of human cancer is not clear, it is known that native ras proto-oncogenes code for the synthesis of nearly 30 different single-chain regulatory proteins (approximate molecular weight of 21,000 daltons each, and, therefore, called "p21" proteins) which bind guanine nucleotides on the cytoplasmic side of the outer cell membrane. Remarkably, the 30 or more ras-related small GTP-binding proteins are highly conserved in structure, whether derived from yeast cells or human tissue cells.

At least three _ras_ proteins (H-_ras_, Ki-_ras_, and N-_ras_) are expressed in most, if not all, mammalian cell types (Bos JL, _Cancer Research_ 49: 4682-4689, 1989).

It is beginning to be appreciated that many of the gene products of proto-oncogenes appear to be involved in particular cell signalling pathways, interacting with one another as important components of an elaborate control network of growth factors, growth-factor receptors, regulators of metabolic pathways, and regulators of chromosomal replication. A delicate balance between these many factors and interactions is no doubt required, not only to maintain the full integrity of a normally-functioning cell, but also in maintaining the active, uncontrolled growth of a malignant cell. A process which causes a major shift in one or more key pathways, then, may be all that is necessary to halt the malignant process, and, perhaps, to initiate events which will rapidly lead to death of the malignant cell. The effectiveness of heterotypic anti-_raf_ antisense oligonucleotide therapy in _ras_-activated cancer may well be dependent on this phenomenon.

The "raf" family of oncogenes.

The _raf_ family of proteins, which have serine/threonine-specific protein kinase activity, are well known as intracytoplasmic signal transducers. There are two known active _raf_ genes in human cells: c-_raf_-1, and A-_raf_-1.

Expressed in all tissues, the c-raf-1 gene is located on chromosome 3p25, in a chromosomal site which has been found to be altered in several epithelial cancers. On the other hand, the A-raf-1 gene, which is located on chromosome Xp11.3, is only expressed in certain tissues. These genes code for cytosolic proteins of approximately 74,000 and 68,000 daltons, respectively.

There is evidence that raf genes function downstream of ras genes in transduction of activation signals from the membrane to the nucleus (Heidecker G., et al., In: Genes and Signal Transduction in Multistage Carcinogenesis (NH Colburn, editor), Marcel Dekker, Inc., New York, pp 352-374, 1989). This conclusion derives from published data which show that antibodies to viral-ras gene, when microinjected into raf-transformed NIH/3T3 cells, do not reverse the transformed state of the raf-transformed cells, whereas microinjection of those same anti-ras antibodies does inhibit the capacity of most other transforming oncogenes to maintain a transformed state of the NIH/3T3 cells. One interpretation consistent with these results suggests that when maintenance of the transformed state of NIH/3T3 cells transformed by a particular oncogene is blocked by microinjection of anti-ras antibodies into those cells, then the transformation-inducing oncogene is not independent of ras gene function and must be positioned in the signal transduction pathway upstream of ras protein function in the cell. On the other hand, dominant oncogenes

(those whose transforming capacity is not affected by blocking ras gene function) must, by similar reasoning, be located downstream of ras in this pathway. It is not known if raf oncogene products are this kind of downstream effector. However, it is known that cellular transformation induced by raf oncogenes is not reversed by microinjection into the raf-activated cell of anti-ras antibodies.

It is believed that this is the first instance of the use of a heterotypic antisense oligonucleotide directed to a non-pathogenic gene to treat disease caused by the expression of an unrelated, mutated disease-causing gene.

BRIEF SUMMARY OF FIGURES

This disclosure is supported by four (4) FIGURES. FIGURE 1 is a histogram drawing showing survival of various NIH/3T3 target cell lines on the left axis plotted against dose of a heterotypic anti-raf antisense oligonucleotide (SEQ ID NO.1) with which the target cells were co-incubated in vitro. FIGURE 2 is made up of 3 smaller sub-figures. FIGURE 2A is a line drawing of cytotoxicity curves, which shows the cytotoxic effect when the heterotypic anti-raf antisense oligonucleotide of the present invention (SEQ ID NO.1) was co-incubated with NIH-3T3 cells. Control experiments are shown in FIGURE 2B and FIGURE 2C, which are drawings of control cytotoxicity curves in which different phosphorothioate oligonucleotides, antisense in sequence to an unrelated gene

(coding for metallothionein). FIGURE 3 is a drawing similar to FIGURE 1, in which a histogram is presented which shows target cell survival on the left axis plotted against dose of the anti-raf oligonucleotide identified as SEQ ID NO.2. FIGURE 4 is a similar drawing in which a histogram is presented which shows survival of the various NIH/3T3 target cells on the left axis plotted against dose of a different heterotypic anti-raf oligonucleotide, identified as SEQ ID NO.3.

DESCRIPTION OF THE INVENTION

In accordance with the present invention there is provided a novel and unexpected method for killing cancer cells expressing an activated ras oncogene by contacting the ras-activated cancer cells in vivo or in vitro with a cytotoxically-effective amount of a heterotypic antisense oligonucleotide or combination of antisense oligonucleotides, or pharmaceutically-effective analogs thereof, which have base sequences complementary to a sequence of the DNA or transcribed messenger RNA of a raf gene also present in the same cancer cells.

By the term "cytotoxically-effective amount", as used herein, is meant an administered amount of a therapeutic oligonucleotide preparation which is well below the cytotoxic endpoint of the oligonucleotide preparation, but which is sufficient to kill ras-activated target cells in preference to other cells.

The present invention also provides novel methods for treating an individual who has _ras_-activated cancer. This treatment involves the use of heterotypic antisense oligonucleotide therapies, in which a cytotoxically-effective amount of a preparation containing an anti-_raf_ antisense oligonucleotide, or combination of selected anti-_raf_ antisense oligonucleotides, or one or more pharmaceutically-effective analogs thereof, is administered as specific drug therapy of cancers expressing an activated _ras_ oncogene. In a preferred embodiment of the present invention, the oligonucleotide preparation is administered systemically to the individual.

It is becoming common to provide cancer-bearing individuals with intensive (potentially lethal) radio- and/or chemotherapy to ablate their tumor burden, followed by rescue with an autologous bone marrow transplant. More recently, rescue with an autologous peripheral stem cell transplant has been performed. However, these transplant procedures will only have long-term value when the autologous transplant cell suspensions are completely free of contaminating tumor cells.

Accordingly, in another embodiment of the present invention, autologous bone marrow cells (or peripheral blood-derived stem cells) from an individual having _ras_-activated cancer are treated _ex vivo_ with specific anti-_raf_ antisense oligonucleotides in order to kill any and all of the _ras_-activated malignant cells which may be contained in the bone

marrow or stem cell transplant specimen. This is a specific improvement over the current procedures being used to deplete contaminating tumor cells from, for example, an autologous marrow or stem cell suspension. After malignant cell depletion, the treated autologous bone marrow cells (or peripheral blood-derived stem cells) are infused back into the patient who has, in the meanwhile, received appropriate surgical, radiation, immuno- and/or chemotherapy.

In the case of an autologous bone marrow transplantation, the method for removing contaminating <u>ras</u>-activated cancerous cells from the marrow cell suspension is straight forward, and comprises the steps of (i) collecting an appropriate amount of bone marrow (preferably about 1500 cc from multiple points in the pelvic iliac crest, although as little as 500 cc and as much as 2000 cc can be used) from the individual who has the <u>ras</u>-activated cancer, and isolating the nucleated cells from the bone marrow sample; (ii) contacting the nucleated bone marrow cells <u>ex vivo</u> (in culture) with a cytotoxically-effective amount of an anti-<u>raf</u> antisense oligonucleotide which has a base sequence complementary to the DNA or transcribed messenger RNA of a <u>raf</u> target gene also present in the cells of the <u>ras</u>-activated cancer (this incubation takes from about 12 hours to about 7 days); and (iii) thereafter infusing the treated bone marrow cells back into the individual patient who donated the marrow. This form of intensive therapy can be further improved by the additional

step of administering systemically to the individual, after the bone marrow transplant has engrafted, a therapeutic preparation of this invention containing anti-raf antisense oligonucleotide, administered in an amount sufficient to kill the few ras-activated cancerous cells which remain in the individual.

The anti-raf antisense oligonucleotides of the present invention can be of significant clinical utility when administered systemically to individuals who have ras-activated cancers, concomitant with or following primary tumor ablation with surgery, radiation and/or chemotherapy. Additional therapeutic gains can be obtained by systemic administration of anti-raf antisense oligonucleotides to recipients of autologous bone marrow cell suspensions, after the bone marrow -- itself purged of contaminating ras-activated cancer cells by treatment with anti-raf oligonucleotides -- has engrafted in the individual.

For effective therapeutic utilization of the novel concepts of the present invention, the anti-raf antisense oligonucleotides are administered *in vivo* as a systemic therapy, and they can also be administered *in vitro*, as a procedure for eliminating contaminating ras-activated tumor cells from a suspension of autologous peripheral blood stem cells or autologous bone marrow cells. Depending on the

intended utilization, the physical form of the therapeutic preparation may vary, as discussed more fully hereinafter.

The size of the oligonucleotide, i.e., the number of bases in the oligonucleotide sequence, is an important consideration. In practice, the length (in base numbers) of a therapeutic oligonucleotide of the present invention ranges from at least about 8 bases to as many as about 50 bases. The longer the antisense oligonucleotide, the higher is its affinity for a target sequence when it binds with an exact complementarity. Furthermore, the longer the antisense sequence being utilized, the more unique is the targeted sequence. However, these advantages are off-set by the fact that the longer oligonucleotides are also more difficult and more costly to prepare and more difficult to handle.

The region of the target DNA or transcribed messenger RNA to which the selected oligonucleotide is designed to hybridize is an important variable that will affect the practice of this invention. Several criteria upon which the targeted region can be selected are: (i) thermal stability of the hybrid complex, which is influenced by the guanine-cytosine (GC) content of the region bound; (ii) secondary structure in the mRNA, such as stem-loops; (iii) regions of intron-exon splicing; and (iv) sequences which are not self-complementary or palindromic.

In addition to these considerations, certain regions of gene sequences are more important for the proper functioning of the gene product than are other regions of that same genome. At least four critical coding regions on the DNA or transcribed messenger RNA must be considered for targeting. They are: (1) the coding region(s) required for initiation of protein synthesis; (2) the coding region(s) which control how the protein will fold in three dimensions; (3) the coding region(s) which code for the active site of the protein; and (4) the coding region(s) which code for termination of the protein synthesis.

First, it is clear that oligonucleotides complementary to a genetic region which includes the initiation coding region of the raf gene are particularly effective in blocking the function of that gene. In a preferred embodiment, therefore, an anti-raf oligonucleotide has a nucleotide base sequence part of which is complementary to the 3-base initiation coding region of the target gene. The most common initiation coding region in the messenger RNA is the 3-base nucleotide sequence AUG, particularly the AUG coding region nearest the 5'-end of the mRNA (usually within 10 to 100 nucleotides of the beginning of the gene). The most common initiation sequence in an mRNA is a 7-base sequence which incorporates the AUG sequence, comprising either AXXAUGG or GXXAUGG, where "X" is any of the four base nucleotides. Much less commonly (about 1 in 30 times) the 3-base sequence GUG is

utilized as an initiation coding region, and, very rarely, the sequences UUG or CUG perform this initiation function. The corresponding complementary sequences in the antisense DNA molecules would then be most commonly TAC, very occasionally CAC, and only rarely AAC or GAC, respectively.

Secondly, the natural protein products of functional genes have a characteristic size and 3-dimen-sional shape, which is dictated by the amino acid sequence coded by the genome. Any significant departure from this natural size and shape usually interferes with the natural 3-dimensional structure of the protein, thereby altering, if not completely abrogating, the function of that modified protein. The experimental process of "denaturing" a protein in a laboratory protocol is an example of how changing the natural 3-dimensional shape of a protein molecule causes it to lose its natural function. Therefore, an antisense oligonucleotide which binds to any portion of the mRNA which codes for an amino acid sequence critical for creating and holding the 3-dimensional shape of the serine/threonine-specific esterase protein molecule will inhibit, if not completely abrogate, the function of that esterase molecule. Likewise, an antisense oligonucleotide which binds to a portion of the DNA or transcribed mRNA which codes for the active site of the _raf_ esterase molecule can also completely block the function of that _raf_ protein.

Fourth, antisense oligonucleotides complementary to a genetic region which includes the termination coding region of the raf gene being targeted will also be effective in modifying, if not completely abrogating, the function of that raf gene. The mRNA coding regions which code for termination of a protein are commonly the 3-base sequences of UAA, UAG, and UGA. Much less commonly the sequences AGA and AGG code for a termination. The corresponding complementary sequences in the antisense DNA molecules would then be ATT, ATC, ACT, TCT, and TCC, respectively. Accordingly, anti-raf oligonucleotides of the present invention have a nucleotide base sequence part of which is complementary to the 3-base termination coding region(s) of the targeted gene sequence.

Anti-raf antisense oligonucleotide selected. As discussed elsewhere herein, there are two known raf genes in the human (see Heidecker et al., In: Genes and Signal Transduction in Multistage Carcinogenesis [NH Colburn, editor], New York: Marcel Dekker, Inc., pp 339-374, 1989). One is designated A-raf-1, and is a gene expressed in select tissues. Located on chromosome Xp11.3, it is approximately 2458 nucleotide bases in length and codes for a cytosolic protein with an approximate molecular weight of 68,000 daltons and having serine/threonine-specific kinase activity. The second raf gene is designated c-raf-1; located on chromosome 3p25, it is expressed in all tissues, is approximately 2977 nucleotide bases in length, and codes for another cytosolic

serine/threonine kinase with an approximate molecular weight of 74,000 daltons.

In practicing the present invention, a number of anti-raf antisense oligonucleotides can be utilized to kill cancer cells which contain an activated ras oncogene. The sequences are herein identified with a SEQUENCE IDENTIFICATION NUMBER, and are antisense to portions of either the A-raf-1 gene, or the c-raf-1 gene, as indicated. Following the SEQUENCE ID NUMBER are given the nucleotide base sequences for each of the anti-raf antisense oligonucleotides, listed in the 5′ to 3′ reading direction for the anti-raf antisense oligo-nucleotide, and, next to each of the antisense sequences, the numerical position of the base sequence in the corresponding portion of the human messenger RNA transcribed from the appropriate human raf gene (where position 1 is the beginning of the gene).

While the anti-raf antisense oligonucleotide sequences of the present invention are described in conjunction with preferred embodiments and specific examples, the listing of these selected sequences is not meant to imply that they are the only ones which may be utilized in practicing this invention. One of ordinary skill in the art, with the aid of the present disclosure, can effect various changes, substitutions of equivalents and other alterations to the methods and compositions herein set forth, in order to practice this invention. For example, one of ordinary skill in

the art can, in order to modify the teaching of the present invention, delete one or more nucleotide bases from a listed anti-*raf* antisense oligonucleotide sequence and retain complete functional capacity to kill the *ras*-activated cancer cells with that modified anti-*raf* oligonucleotide; in practicing this invention, one can shorten the antisense oligonucleotide by deleting nucleotide bases from the listed sequence until the killing function in an *in vitro* assay is lost. Similarly, one of ordinary skill in the art can modify the teaching of the present invention by lengthening the anti-*raf* antisense oligonucleotide by adding one or more nucleotide bases to a listed anti-*raf* oligonucleotide sequence and possibly retain funtional capacity to kill the *ras*-activated cancer cells with the longer anti-*raf* (modified) oligonucleotide.

1. SEQ ID NO.1:

(nucleotide sequence of antisense oligo EC-1C)
5′ C T G T T G G A A A T C C T A G A A 3′

Site: 1269 through 1252 of the human mRNA for c-*raf*-1 oncogene.

2. SEQ ID NO.2:

(nucleotide sequence of antisense oligo EC-2C):
5′ T C T T G G T G A G G T C G C A C T 3′

Site: 1626 through 1609 of the human mRNA for c-*raf*-1 oncogene.

3. SEQ ID NO.3:

(nucleotide sequence of antisense oligo EC-3C):
5′ C A A G A A T A T A T C G A A T G A 3′

Site: 1857 through 1840 of the human mRNA for c-<u>raf</u>-1 oncogene.

4. SEQ ID NO.4:

(nucleotide sequence of antisense oligo DK-1)
5′ G G C G G G G T T G C A G G A C A G 3′

Site: 89 through 72 of the human mRNA for c-<u>raf</u>-1 oncogene.

5. SEQ ID NO.5:

(nucleotide sequence of antisense oligo DK-2)
5′ G T T T T G G T A A C G A C T A G 3′

Site: 175 through 158 of the human mRNA for c-<u>raf</u>-1 oncogene.

6. SEQ ID NO.6:

(nucleotide sequence of antisense oligo DK-3)
5′ C T C G G T A G T T T G T 3′

Site: 203 through 191 of the human mRNA for c-<u>raf</u>-1 oncogene.

7. SEQ ID NO.7:

(nucleotide sequence of antisense oligo DK-4)
5′ T C A A A C G G T A G T A G A C T A 3′

Site: 272 through 255 of the human mRNA for c-<u>raf</u>-1 oncogene.

8. SEQ ID NO.8:

(nucleotide sequence of antisense oligo DK-5)
5′ G T G G A A C T C A C G A A A G T A T T
C C G T C A G T A C G T T C G A G T A A
G G T A A A G C G T 3′

Site: 393 through 341 of the human mRNA for c-<u>raf</u>-1 oncogene.

9. SEQ ID NO.9:

(nucleotide sequence of antisense oligo DK-6)
5' C T G C G T C G T A G T C 3'

Site: 488 through 476 of the human mRNA for c-raf-1 oncogene.

10. SEQ ID NO.10:

(nucleotide sequence of antisense oligo DK-7)
5' C G A T G T C A C G A G T A C T T T 3'

Site: 660 through 643 of the human mRNA for c-raf-1 oncogene.

11. SEQ ID NO.11:

(nucleotide sequence of antisense oligo DK-8)
5' A A C G T A G G A G T T A G T A G G A C 3'

Site: 970 through 951 of the human mRNA for c-raf-1 oncogene.

12. SEQ ID NO.12:

(nucleotide sequence of antisense oligo DK-9)
5' G T A G T G A A G T G A C C G A A G A T 3'

Site: 1179 through 1159 of the human mRNA for c-raf-1 oncogene.

13. SEQ ID NO.13:

(nucleotide sequence of antisense oligo DK-10)
5' A C G G T A A A T G G G A A T 3'

Site: 1235 through 1221 of the human mRNA for c-raf-1 oncogene.

14. SEQ ID NO.14:

(nucleotide sequence of antisense oligo DK-11)
5' C G G T G G A G T A A G G A C T T C 3'

Site: 1313 through 1296 of the human mRNA for c-raf-1 oncogene.

15. SEQ ID NO.15:

(nucleotide sequence of antisense oligo DK-12)
5' A G G A A A A C A G T A C A T G G G G T A 3'

Site: 1373 through 1354 of the human mRNA for c-raf-1 oncogene.

16. SEQ ID NO.16:

(nucleotide sequence of antisense oligo DK-13)
5' T T G T A G A C 3'

Site: 1457 through 1449 of the human mRNA for c-raf-1 oncogene.

17. SEQ ID NO.17:

(nucleotide sequence for antisense oligo DK-14)
5' G T A C G T T T A T C A G G T A A G G G
A C T C 3'

Site: 1510 through 1487 of the human mRNA for c-raf-1 oncogene.

18. SEQ ID NO.18:

(nucleotide sequence for antisense oligo DK-15)
5' G G G C A G T A G T C A A G T A T G T T 3'

Site: 1760 through 1741 of the human mRNA for c-raf-1 oncogene.

19. SEQ ID NO.19:

(nucleotide sequence for antisense oligo DK-16)
5' T C G A T G G T C G G A G A A G T A A C
G A A A C C C C G T C A A G A A T A T A
T C G A A T G A T T 3'

Site: 1887 through 1838 of the human mRNA for c-raf-1 oncogene.

20. SEQ ID NO.20:

(nucleotide sequence for antisense oligo DK-17)
5' A C C C C T T T T T C T C C G G A G A G
A A G G A A A T G A A A G A A G T G T 3'

Site: 1931 through 1893 of the human mRNA for c-raf-1 oncogene.

21. SEQ ID NO.21:

(nucleotide sequence for antisense oligo DK-18)
5' T T T C C C T C G T C T T T T C A C C A C G G A 3'

Site: 2149 through 2126 of the human mRNA for c-<u>raf</u>-1 oncogene.

22. SEQ ID NO.22:

(nucleotide sequence for antisense oligo DK-19)
5' G A C G T T T A C C G A A G G A A G 3'

Site: 2278 through 2261 of the human mRNA for c-<u>raf</u>-1 oncogene.

23. SEQ ID NO.23:

(nucleotide sequence for antisense oligo DK-20)
5' G A T G G A A T G A A G G A G A T T T A 3'

Site: 2425 through 2406 of the human mRNA for c-<u>raf</u>-1 oncogene.

24. SEQ ID NO.24:

(nucleotide sequence for antisense oligo DK-21)
5' G G G G A T T C T T T T C A A G G T A T C A T G G T 3'

Site: 2626 through 2601 of the human mRNA for c-<u>raf</u>-1 oncogene.

25. SEQ ID NO.25:

(nucleotide sequence for antisense oligo DK-22)
5' T T T T G G T A G G G T T 3'

Site: 2678 through 2666 of the human mRNA for c-<u>raf</u>-1 oncogene.

26. SEQ ID NO.26:

(nucleotide sequence for antisense oligo DK-23)
5′ C A A A C A A A C A A A C A A A C A A T
C 3′

Site: 2819 through 2798 of the human mRNA for c-raf-1 oncogene.

27. SEQ ID NO.27:

(nucleotide sequence for antisense oligo DK-24)
5′ G G T A G A A C 3′

Site: 106 through 99 of the human mRNA for A-raf-1 oncogene.

28. SEQ ID NO.28:

(nucleotide sequence for antisense oligo DK-25)
5′ C G G T A G G G 3′

Site: 308 through 301 of the human mRNA for A-raf-1 oncogene.

29. SEQ ID NO.29:

(nucleotide sequence for antisense oligo DK-26)
5′ A G G T A G T G A T G G A G G A G A T G
A T G 3′

Site: 950 through 928 of the human mRNA for A-raf-1 oncogene.

30. SEQ ID NO.30:

(nucleotide sequence for antisense oligo DK-27)
5′ T A G C G G T A C G G T G G C G G G 3′

Site: 1190 through 1173 of the human mRNA for A-raf-1 oncogene.

31. SEQ ID NO.31:

(nucleotide sequence for antisense oligo DK-28)
5′ G A C C T G G T A C A G C T T C G C 3′

Site: 1409 through 1392 of the human mRNA for A-raf-1 oncogene.

32. SEQ ID NO.32:

(nucleotide sequence for antisense oligo DK-29)

5′ C T C C A T C A G G T A C G G G A C C C
G 3′

Site: 1454 through 1434 of the human mRNA for A-raf-1 oncogene.

33. SEQ ID NO.33:

(nucleotide sequence for antisense oligo DK-30)
5′ T T G A G T A G C C 3′

Site: 1975 through 1966 of the human mRNA for A-raf-1 oncogene.

34. SEQ ID NO.34:

(nucleotide sequence for antisense oligo DK-31)
5′ C G T C G T A G T C C C G 3′

Site: 2110 through 2098 of the human mRNA for A-raf-1 oncogene.

35. SEQ ID NO.35:

(nucleotide sequence for antisense oligo DK-32)
5′ G T A C C C C T G G G G G A G T A G A G
G G T C C C A C C C C T T A C C C C C 3′

Site: 2159 through 2119 of the human mRNA for A-raf-1 oncogene.

36. SEQ ID NO.36:

(nucleotide sequence for antisense oligo DK-33)
5′ G G G T T T T A A A T C T T C A 3′

Site: 2266 through 2251 of the human mRNA for A-raf-1 oncogene.

37. SEQ ID NO.37:

(nucleotide sequence for antisense oligo DK-34)
5′ C T T C C G T G T A G T C C G T G T 3′

Site: 2348 through 2331 of the human mRNA for A-raf-1 oncogene.

Anti-raf oligonucleotides for systemic administration

Nuclease-resistant backbone structure in the preferred embodiment of the present invention. The "normal" structure of an oligonucleotide is a defined sequence of nucleotide bases built upon a sugar-phosphate backbone containing phosphodiester linkages. However, considerable experience indicates that the normal phosphodiester linkage is highly susceptible to rapid degradation by a variety of nucleases found in abundance in tissues and cellular fluids. For an antisense oligonucleo-tide to be useful as a therapeutic agent following systemic administration, it must survive in solution long enough to reach its designated target gene in the body and block the activity of that target gene.

Therefore, in preferred embodiments of the present invention, the anti-*raf* antisense oligonucleotides are those analogs which contain a nuclease-resistant backbone linkage structure. A number of these nuclease-resistant linkage structures are known in the art (for example, see the discussion of nuclease-resistant linkages in: Stein CA, et al., Nucleic Acids Research 16: 3209-3221, 1988). One such linkage is the phosphorothioate linkage. Phosphorothioates are compounds well known in the art, and are those in which one of the non-bridging oxygen atoms in the phosphate portion of a nucleotide is replaced by sulfur. The use of oligo-nucleotide analogs which contain a backbone of phophorothioate

linkages is based on the known resistance of this internucleotide linkage to degradation by nucleases of many types. Since phosphorothioates also have the same number of charges as normal phosphodiester-linked oligonucleotides, they have good aqueous solubility.

Antisense phosphorothioate analogues have been used by several groups in assays for measuring antisense activity, and evidence indicates that this nuclease-resistant backbone linkage does not diminish the potential for sequence specific recognition by the oligonucleotide analog of its target gene. Furthermore, the unmodified (normal) oligonucleotide has a half-life in vivo of about 2 hours, whereas more than 95% of the phophorothioate bonds are still intact after 10 days in vivo.

In addition to the preferred phosphorothioate linkage, the antisense oligonucleotides selected for practice of the invention may have nuclease-resistant ethyl- or methylphosphonate linkages between nucleotide bases. However, experience has shown that oligonucleotide analogs with these types of linkages are less efficient at hybridization with a complementary DNA sequence than are the corresponding analogs which incorporate phorphorothioate linkages. On the other hand, oligonucleotides having a methylphosphonate backbone are more lipophilic than are the other analogs, and this may prove advantageous in certain circumstances. For example, ribozyme

structures (Greene JJ, Clinical Biotechnology 2: 75-76, 1990), incorporating methyl-phosphonate oligonucleotide analogs, have a long half-life *in vivo* because the lipophilic structure reduces the rate of renal clearance of the compound while the ribozyme structure facilitates cleavage of the target RNA message (Gerlach, Nature 334: 585, 1988).

To those skilled in the art, it is known that nuclease-resistant backbone linkages other than those mentioned above are readily available for incorporation into all or part of a newly-synthesized oligonucleotide. Furthermore, it is also known that other nuclease-resisting linkages are continually being being developed. It is the intent of the present invention that any anti-*raf* antisense oligonucleotide used alone or in combination with other therapies, and which contains such nuclease-resistant backbone linkages be included within the scope of the present invention.

Use of anti-raf antisense oligonucleotides in pharmaceutical formulations. To be available for use in systemic administration, the therapeutic anti-*raf* antisense oligonucleotides must be formulated into suitable pharmaceutical compositions; the protocol for systemic administration would use a therapeutic approach compatible with the particular formulation selected. Pharmaceutical compositions within the scope of the present invention include those compositions where the anti-*raf* oligonucleotide is

contained in an effective amount sufficient to kill the *ras*-activated cells of the cancer without causing unacceptable toxicity for the patient. The therapeutic amount which represents a cytotoxically-effective dose sufficient for treatment of each of the various types of *ras*-activated tumor remains to be determined empirically by those skilled in the art of designing and administering chemotherapy. However, a preferred dosage comprises that which is sufficient to achieve an effective blood concentration of from about 0.1 to about 200 micromolar.

The anti-*raf* antisense oligonucleotide compounds of the present invention (also referred to hereinafter as the "active ingredients" or "active compounds"), in whatever analog prepared, may be administered in a pharmaceutical composition which contains, in addition to the active ingredient, any of a number of pharmaceutically-acceptable excipients which facilitate processing of the active compound into suitable pharmaceutical preparations. In a preferred embodiment, the preparations are designed for parenteral administration. However, pharmaceutical compositions designed for oral administration in such forms as tablets, capsules, and dragees, or for rectal administration in the form of suppositories, are also considered to fall within the scope of the present invention.

Appropriate formulations of the therapeutic oligonucleotide for parenteral administration include aqueous solutions of the active compound prepared in a water-soluble or water-dispersible form. Alternatively, the active compounds may be administered as suspensions in appropriate oily injection carriers, i.e., in suitable lipophilic carriers, such as fatty oils (sesame oil being an example), or synthetic fatty acid esters (ethyl oleate or triglycerides being examples). Pharmaceutical formulations prepared for aqueous injection may contain substances which increase the viscosity of the suspension such as, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran.

The therapeutic anti-_raf_ oligonucleotides of the present invention may also be administered encapsulated in liposomes. In such pharmaceutical preparations, the anti-_raf_ oligonucleotides are contained in corpuscles which consist of concentric aqueous layers interspersed between hydrophobic lipidic layers. The oligonucleotides, depending upon their solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such as a diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature which are generaly well known in the art.

Antisense oligonucleotide treatment of bone-marrow cells

Purging bone marrow suspensions of contaminating tumor cells is presently accomplished either by *in vitro* incubation of the transplanted marrow cells with potent anti-cancer chemotherapeutic agents, or by contacting the bone marrow cells with immunotherapeutic agents which recognize certain structures unique to the surface membrane of tumor cells.

A major difficulty with immunotherapy is the fact that many tumor cells fail to express the tumor-associated membrane structure, and thereby go unrecognized by the immunotherapeutic agent. On other tumor cells, the immunotherapeutic agent binds to its target but fails to kill the cell. With regard to chemotherapetic agents, most of the agents are highly toxic and must be used at relatively high doses in order to maximize tumor cell kill. However, this can lead to death of a large number of normal marrow cells and, in some instances, to graft failure. What is needed, therefore, is a bone marrow purging agent which selectively kills tumor cells and leaves the normal marrow cells intact. The present invention provides such an agent for use with *ras*-activated cancers.

Thus, in another embodiment of the present invention, anti-*raf* antisense oligonucleotides are used to kill any and all *ras*-activated cancer cells which may be present in a suspension of bone marrow cells obtained from the afflicted

individual. In this latter technique, bone marrow cells are obtained from an individual who has a ras-activated cancer, using standard procedures, which include aspiration from the pelvic iliac crest of a donor, as described, for example, in U.S. Patents No. 4,481,946 and No. 4,486,188. The patient from whom the bone marrow has been taken is then treated with radiation or chemotherapy to destroy the ras-activated cancer cells which are in one or more organs of the body. Because this intensive therapy also destroys sensitive stem cells required for re-establishment and regrowth of such vital systems as the hematopoietic system, the treated patient must be replenished with healthy autologous bone marrow cells. Clearly, it is to the long-term advantage of the patient if the bone marrow cells returned to the patient are entirely free of cancer cells. These and other potential concerns are discussed in detail in: Autologous Bone Marrow Transplantation: Proceedings of the Third International Symposium, K. Dicke (editor), The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, 1987.

The sample of autologous bone marrow cells is then immediately treated with the anti-raf oligonucleotide, as discussed below, and reinfused into the donor as soon as is appropriate. In such a treatment, the autologous bone marrow is purged of contaminating cancer cells by exposure ex vivo to a cytotoxically-effective amount of an antisense oligonucleotide which has a nucleotide sequence complementary to that of

an RNA message transcribed from a raf target gene present in the cells of the ras-activated cancer.

The time of exposure required to obtain complete kill of the targeted cells in the bone marrow specimen varies depending on the tumor cell target and must be determined empirically; however, exposure times varies from 1 hour to 4 days or longer. Following exposure to the therapeutic anti-raf oligonucleotide preparation, the autologous bone marrow purged of all ras-activated malignant cells is transplanted back into the donor.

Alternatively, if the opportunity or need to use the oligonucleotide-treated marrow sample is not immediate, the purged bone marrow cells can be frozen and stored until needed. Procedures for preparing and storing bone marrow samples frozen in a viable state are discussed in detail in U.S. Patents No. 4,107,937 and No. 4,117,881.

Antisense oligonucleotide treatment of peripheral blood-derived stem cells.

There are in the circulating peripheral blood a substantial number of mononuclear cells which have the potential to regenerate the complete function of the bone marrow compartment of a host organism, such as a human. These peripheral "stem" cells can be isolated, concentrated, and

reintroduced via injection into the peripheral circulation as a "stem cell transplant."

Autologous peripheral blood stem cell transplantation has been found important in facilitating recovery of functional bone marrow after high-dose therapy for a variety of malignant diseases. Autologous peripheral blood stem cell transplantation offers certain advantages to autologous bone marrow transplantation, since the general anesthesia used during bone marrow harvesting can be avoided, the collections of peripheral stem cells can be made in an outpatient setting, and the risk of contamination of the transplanted product with malignant cells appears to be less.

Purging the peripheral stem cell suspension of contaminating tumor cells are very similar, if not identical, to the procedures outlined above for purging bone marrow cells with anti-raf antisense oligonucleotides.

It is impossible to determine, prior to a patient's receiving the autologous bone marrow or peripheral stem cell transplant, whether a series of radiotherapy or chemotherapy treatments has completely rid that patient of all ras-activated malignant cells. Therefore, another embodiment of the present invention is to provide a course of systemically-administered antisense oligotherapy as an adjunct therapy to

the individual who received the transplant of autologous bone marrow cells or peripheral stem cells.

The following examples more fully demonstrate the present invention.

EXAMPLE 1

Use of anti-raf antisense oligonucleotide SEQ ID NO.1 for killing ras-activated tumor target cells

Cell lines and culture. NIH/3T3 mouse embryo fibroblasts (ATCC Culture # CCL-92, American Type Culture Collection, Rockville, MD), as well as several NIH/3T3 transformants and virally infected cell lines, were grown in Dulbecco-modified Eagle's minimal essential medium supplemented with 10 percent (volume/volume) heat-inactivated calf serum, 50 ug/ml each of gentamicin sulfate antibiotic, and exogenous glutamine to a final concentration equivalent to 2 mmol per liter.

Transfections. Transfections were carried out by standard calcium phosphate precipitation method. High molecular weight DNA (25 ug/75 cm^2 flask) was co-transfected with pSV_2 neo plasmid (600 ng/75 cm^2 flask) into NIH/3T3 cells and selected for capacity to grow in the presence of G418. Resistant cells were propagated without prior clonal selection and injected (300 colonies/mouse) into 6-to-8 week old female

athymic nude mice. Morphological transformation of the NIH/3T3 cells was not a selection criterion before inoculation into nude mice. Tumor-bearing animals were sacrificed when the tumor was 0.5-1 cm in diameter. High molecular weight DNA was obtained from tumors and analyzed for the presence of human sequences.

Six different long-term cultured NIH/3T3 cell lines were incubated for 24 hours in the presence of an anti-raf antisense oligonucleotide, designated SEQ ID NO.1 (also referred to as "EC-1C" in the laboratory experiments). The nucleotide base sequence is complementary to a portion of the mRNA transcribed from the c-raf-1 oncogene, site positions 1269-1252, as follows:

SEQ ID NO.1: 5′ C T G T T G G A A A T C C T A G A A 3′

The oligonucleotide was constructed with a phosphorothioate backbone, as described hereinafter.

Oligonucleotide synthesis and purification. Oligonucleotide EC-1C (SEQ ID NO.1) was synthesized on an Applied Biosystems Model 380A DNA synthesizer. Each synthetic cycle resulted in a phosphite linkage which was oxidized with I_2/H_2O/base to a phosphotriester precursor of an unmodified linkage or was sulfurized with S_8/CS_2/pyridine to make a phosphorothioate linkage. Analysis of dimethoxytrityl removal

per synthetic step provided an initial detection and diagnosis of synthesis problems. The "deprotected" oligonucleotide was precipitated from ethanol, the pellet was resuspended and desalted over C-18 reverse-phase cartridges. Final purification was accomplished by reverse-phase HPLC with an increasing gradient of acetonitrile in triethylammonium acetate or by HPEC separation in triethylammonium acetate. The purified product was quantified by determination of the absorbance at 260 nm employing a weighted molar extinction coefficient based on nucleotide composition.

Cytotoxicity Assay. Eight different concentrations of SEQ ID NO.1 ("EC-1C") anti-*raf* oligonucleotide, prepared by serial 1:1 dilutions in the wells of 96-well microtest plates, were examined for their capacity to produce death of the cell lines with which it had been contacted *in vitro* for 24 hours. Cell death was determined by the loss of the ability of cell mitochondria to reduce MTT dye. This is a quantitative colorimetric assay for mammaliam cell survival and proliferation, employing MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide). Only living cells with active mitochondria can reduce MTT, a process which generates a colored formazan dye. A Molecular Devices 96-well plate reader was used to conduct this assay, permitting an entire oligonucleotide dose response curve to be conducted within several hours.

Results. The results are shown in FIGURE 1. FIGURE 1 is a drawing in which a histogram is presented. The drawing shows cell survival on the left axis plotted against dose of the anti-raf oligonucleotide. The data were analyzed by fitting the log dose of oligonucleotide versus percent cell survival to a sigmoid curve. The LD-50 (a measure of potency) values are presented: open bars, NIH/3T3 control (untreated) cells; right diagonal hatching, NIH/3T3 cells with a "normal" ras proto-oncogene stably inserted into the cellular genome; left diagonal hatching, NIH/3T3 cells (also designated "S80" cells) with "activated" Harvey-ras-1 oncogene sequence stably inserted into the cellular genome; horizontal hatching, NIH/3T3 ("453") cells; vertical hatching, NIH/3T3 ("485") cells; and cross hatching, NIH/3T3 ("504") cells. The 504 cells were more sensitive to the SEQ ID NO.1 oligonucleotide than were the other cells, but were more easily washed off the plates during the assay than the other cells.

As shown in FIGURE 1, the SEQ ID NO.1 phosphorothioate oligonucleotide with sequence antisense to a select portion of the oncogene c-raf-1 was found to be greater than ten times more lethal in NIH/3T3 mouse fibroblast cells which expressed activated ras than in the NIH/3T3 cells which contained normal ras proto-oncogene. This is also shown in another manner in FIGURE 2A. FIGURE 2A is a drawing of cytotoxicity curves. The triangles represent NIH/3T3 murine cells which show 60% cell survival at 1 uM dose, the open circles represent NIH/3T3

with the human <u>ras</u> proto-oncogene stably integrated and show 55% cell survival at 1 uM dose, and the closed circles represent NIH/3T3 cells with activated human <u>ras</u> oncogene stably integrated and show only 5% cell survival at 1 uM dose.

Control experiments are shown in FIGURE 2B and FIGURE 2C, which are also drawings of cytotoxicity curves. In these drawings, the different phosphorothioate oligonucleotides antisense in sequence to the gene coding for metallothionein all exert the same level of cytotoxicity against the three NIH/3T3 cell types. This demonstrates that the phosphorothioate backbone is not responsible for selective cell cytotoxicity. The final control shown in the drawing in FIGURE 2C shows the diminished cytotoxicity of the antisense metallothionein when used as a double-stranded oligonucleotide. These data, taken together, indicate that it is possible to selectively kill cells containing an activated <u>ras</u> oncogene by using an anti-<u>raf</u> antisense oligonucleotide.

<u>EXAMPLE 2</u>

<u>Use of anti-raf antisense oligonucleotide SEQ ID NO.2 for killing ras-activated tumor target cells</u>

Six different long-term cultured NIH/3T3 cell lines were incubated for 24 hours in the presence of an anti-<u>raf</u> antisense oligonucleotide, designated SEQ ID NO.2 (also referred to as "EC-2C" in the laboratory experiments). The nucleotide base sequence is complementary to a portion of the

mRNA transcribed from the c-raf-1 oncogene, site positions 1626-1609, as follows:

SEQ ID NO.2: 5′ T C T T G G T G A G G T C G C A C T 3′

The oligonucleotide was constructed with a phosphorothioate backbone, as described hereinafter.

SEQ ID NO.3: 5′ C A A G A A T A T A T C G A A T G A 3′

Cytotoxicity Assay. In a manner similar to that described above in Example 1, eight different concentrations of SEQ ID NO.2 ("EC-2C") anti-raf oligonucleotide were prepared by serial 1:1 dilutions in the wells of 96-well microtest plates, and examined for their capacity to produce death of the cell lines co-cultured in vitro with the anti-raf antisense oligonucleotide for 24 hours. Cell death was determined by the loss of the ability of cell mitochondria to reduce MTT dye, as outlined above in Example 1.

Results. The results are shown in FIGURE 3. FIGURE 3 is a drawing in which a histogram is presented. The drawing shows cell survival on the left axis plotted against dose of the anti-raf oligonucleotide. The data were analyzed by fitting the log dose of oligonucleotide versus percent cell survival to a sigmoid curve. The LD-50 (a measure of potency) values are presented in the figures: the different vertical

bars in the histogram represent the same target cell types as described in the histogram of FIGURE 1.

As seen in FIGURE 3, the SEQ ID NO.2 phosphorothioate oligonucleotide with an antisense nucleotide sequence uniquely complementary to a select portion of the oncogene c-<u>raf</u>-1 were found to be about eight times more lethal in NIH/3T3 mouse fibroblast cells with activated <u>ras</u> expressed than in the NIH/3T3 cells which contained normal <u>ras</u> proto-oncogene.

<u>EXAMPLE 3</u>

<u>Use of anti-raf antisense oligonucleotide SEQ ID NO.3 for killing ras-activated tumor target cells</u>

Six different long-term cultured NIH/3T3 cell lines were incubated for 24 hours in the presence of an anti-<u>raf</u> antisense oligonucleotide, designated SEQ ID NO.3 (also referred to as "EC-3C" in the laboratory experiments). The nucleotide base sequence is complementary to a portion of the mRNA transcribed from the c-<u>raf</u>-1 oncogene, site positions 1857-1840, as follows:

SEQ ID NO.3: 5' C A A G A A T A T A T C G A A T G A 3'

The oligonucleotide was constructed with a phosphorothioate backbone, as described hereinafter.

Cytotoxicity Assay. In a manner similar to that described above in Example 1, eight different concentrations of SEQ ID NO.3 ("EC-3C") anti-raf oligonucleotide were prepared by serial 1:1 dilutions in the wells of 96-well microtest plates, and examined for their capacity to produce death of the cell lines co-cultured in vitro with the anti-raf antisense oligonucleotide for 24 hours. Cell death was determined by the loss of the ability of cell mitochondria to reduce MTT dye, as outlined above in Example 1.

Results. The results are shown in FIGURE 4. FIGURE 4 is a drawing in which a histogram is presented. The drawing shows cell survival on the left axis plotted against dose of the anti-raf oligonucleotide. The data were analyzed by fitting the log dose of oligonucleotide versus percent cell survival to a sigmoid curve. The LD-50 (a measure of potency) values are presented in the figures: the different vertical bars in the histogram represent the same target cell types as described in the histogram of FIGURE 1.

As seen in FIGURE 4, the SEQ ID NO.3 phosphorothioate oligonucleotide with an antisense nucleotide sequence uniquely complementary to a select portion of the oncogene c-raf-1 were found to be about twenty times more lethal in NIH/3T3 mouse fibroblast cells with activated ras expressed than in the NIH/3T3 cells which contained normal ras proto-oncogene.

EXAMPLE 4

Use of anti-raf antisense oligonucleotides in autologous peripheral blood stem cell transplantation

As described above, the anti-*raf* antisense oligonucleotides of the present invention may be administered as systemic oligotherapy, or it may be used *in vitro* to purge contaminating tumor cells from an autologous peripheral blood stem cell preparation to be used in an autologous transplantation procedure. The following prophetic example illustrates how the anti-*raf* oligonucleotides of the present invention can be used to purge contaminating *ras*-activated cancer cells from a suspension of peripheral blood stem cells.

Using procedures well known in the art (for example, see: Kessinger et al., Blood 74: 1260-1265, 1989), the peripheral stem cells are collected with a Haemonetics Model V50 apheresis device (Haemonetics, Braintree, MA). The stem cells are collected in a technique called a component collection lymphocytopheresis autosurge protocol described in the operating manual for the apheresis instrument. Venous blood is withdrawn into a Latham apheresis bowl. Centrifugation is continued until the layer of red blood cells reaches a set point on the shoulder of the bowl. Then the collected plasma is rapidly pumped back into the bowl, resulting in elutriation of mononuclear cells. The remaining red blood cells, platelets, granulocytes, and plasma are

returned to the patient. One collection consists of repeating the procedure for four hours.

The mononuclear cell fraction is then further fractionated in the apheresis device using a Ficoll-diazitroate density gradient to remove contaminating red blood cells. The red cell depleted product is washed twice by centrifugation and resuspended in Hank's balanced salt solution without calcium or magnesium with 20% (volume/volume) autologous serum and 0.6% citrate formula B (Fenwal, Deerfield, IL).

Approximately eight collections are made to harvest the stem cells used for a patient's stem-cell transplant. All stem cell collected are cryopreserved in a buffered salt solution containing a 10% concentration of dimethylsulfoxide (DMSO) as a cryoprotectant.

Following therapy, at a time which is clinically appropriate for the patient to receive the autologous transplant, the stem cells are thawed, warmed to normal body temperature (37°C), and infused intravenously.

The separated peripheral stem cells are then resuspended at a concentration of approximately 10^7 cells per ml in a culture medium made up of the following: RPMI 1640 balanced salt solution, supplemented with a source of growth

factors (such as "5637 Conditioned Medium"); 5 uM per liter hydrocortisone hemisuccinate; 250 ug per ml catalase; 2 mM per liter mannitol; 1% (volume/volume) of a 100X sodium pyruvate solution; 1% (v/v) of a 100X vitamin solution; 1% (v/v) of a 50X amino acid solution; 0.5% (v/v) of a 200X nonessential amino acids solution; L-glutamine to a final concentration of 200mM per liter. To this is then added autologous, sterile-filtered autologous serum to a final concentration of 20%. Finally, an anti-raf antisense oligonucleotide or combination of anti-raf antisense oligonucleotides is added to a concentration of approximately 50 uM.

This suspension of peripheral stem cells in nutrient medium, in the presence of anti-raf antisense oligonucleotide, is then incubated in culture vessels at 37°C in 5% CO_2 in humidified air for from 2 hr to 5 days. At appropriate and regular intervals of time, the cultured cells are fed by a change of 2/3 of the medium, and by a dilution of the cells into additional culture vessels to near the original cell density. The presence of progenitor stem cells is determined in assays for Colony-Forming Units - Granulocyte/Macrophage (CFU-GM), and assays for Blast-Forming Units - Erythrocyte (BFU-E).

The stem cell cultures are harvested when it is determined that no ras-activated tumor cells remain among the

peripheral stem cells. Using radiolabeled probes to the ras oncogene and polymerase chain reaction (PCR) amplification of test samples, this determination is rapid and accurate. The treated stem cells are then concentrated and transferred into infusion bags, where they are infused back into the donor.

The recipient of the autologous transplant is then followed to verify that the transplanted cells have successfully engrafted in the host marrow. When this has been confimed, additional anti-raf antisense oligonucleotide is administered systemically to the patient in a low dose maintenance shcedule, as discussed above, to be certain that any remaining ras-activated tumor cells in the patient is eliminated. This pharmaceutical preparation contains one or more of the anti-raf antisense oligonucleotides described as useful in the present invention.

EXAMPLE 5

Use of anti-raf antisense oligonucleotides in autologous bone marrow transplantation

As described above, the anti-raf antisense oligonucleotides of the present invention may be administered as systemic oligotherapy, or used in vitro to purge tumor cells from an autologous bone marrow preparation. The following prophetic example illustrates how the anti-raf oligonucleotides of the present invention can be used to purge contaminating ras-activated cancer cells from a suspension of bone marrow cells.

Using procedures well known in the art, a total of approximately 1500 ml of bone marrow cell suspension is aspirated from several different points of the posterior iliac crest of the donor's pelvic bone. The heparin-treated cell suspension is then aseptically transferred to sterile centrifuge tubes, in which large aggregates and bone spicules are allowed to settle for several minutes, after which the cell suspension is centrifuged over a density gradient such as Ficoll-Diazitroate (density approximately 1.077 to 1.079) to separate the less-dense mononuclear cells of the marrow from the more dense red cells and granulocytes. Alternatively, by selective unit-gravity sedimentation in a density solution such as dextran, the red cells only are removed and all nucleated cells are retained for further workup.

The separated bone marrow cells are then resuspended at a concentration of approximately 107 cells per ml in a culture medium made up of the following: RPMI 1640 balanced salt solution, supplemented with a source of growth factors (such as "5637 Conditioned Medium"); 5 uM per liter hydrocortisone hemisuccinate; 250 ug per ml catalase; 2 mM per liter mannitol; 1% (volume/volume) of a 100X sodium pyruvate solution; 1% (v/v) of a 100X vitamin solution; 1% (v/v) of a 50X amino acid solution; 0.5% (v/v) of a 200X nonessential amino acids solution; L-glutamine to a final concentration of 200mM per liter. To this is then added ultra-filtered horse serum (Hyclone Laboratories, Logan, Utah) to a final

concentration of 12.5%, and ultra-filtered fetal calf serum (Hyclone) to 12.5% as well. Finally, anti-<u>raf</u> antisense oligonucleotide is added to a concentration of approximately 50 uM.

This suspension of bone marrow cells in nutrient medium, in the presence of antisense oligonucleotide, is then incubated in culture vessels at 37oC in 5% CO2 in humidified air. At appropriate and regular intervals of time, the cultured cells are fed by a change of 2/3 of the medium, and by a dilution of the cells into additional culture vessels to near the original cell density.

The cultures are harvested when it is determined that no <u>ras</u>-activated tumor cells remian in the bone marrow culture. Using radiolabeled probes to the <u>ras</u> oncogene and polymerase chain reaction (PCR) amplification of test samples, this determination is rapid and accurate. The treated marrow cells are then concentrated and transferred into infusion bags, where they are infused back into the donor.

The recipient of the autologous transplant is then followed to verify that the marrow has successfully engrafted. When this has been confirmed, additional anti-<u>raf</u> antisense oligonucleotide is administered systemically to the patient in a low dose maintenance shcedule, as discussed above, to be certain that any remaining <u>ras</u>-activated tumor cells in the

patient are eliminated. This pharmaceutical preparation contains one or more of the anti-raf antisense oligonucleotides described as useful in the present invention.

* * * * * * * *

While the present invention has been described in conjunction with preferred embodiments and specific examples, the description is not meant to limit it. One of ordinary skill, with the aid of the present disclosure, may be able to effect various changes, substitutions of equivalents and other alterations to the methods and compositions herein set forth. Therefore, the protection granted by Letters Patent should not be limited except by the language of the claims as set forth below.

"<u>SEQUENCE LISTING</u>"

[1] <u>GENERAL INFORMATION</u>
[i] APPLICANT: IVERSEN, Patrick L., Ph.D.

[ii] TITLE OF INVENTION: "Treatment of <u>Ras</u>-Activated Cancer with <u>Raf</u> Antisense Oligonucleotide Probes"

[iii] NUMBER OF SEQUENCES: thirty-seven (37)

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[v] COMPUTER READABLE FORM:
[A] MEDIUM TYPE: floppy disk, 5.25 inch, 360 Kb storage
[B] COMPUTER: IBM-compatable type
[C] OPERATING SYSTEM: MS-DOS 4.1
[D] SOFTWARE: WordPerfect 5.0

[vi] CURRENT APPLICATION DATA:
[A] APPLICATION NUMBER: not available
[B] FILING DATE: not available
[C] CLASSIFICATION: not available

[vii] PRIOR APPLICATION DATA: none

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[2] <u>INFORMATION FOR SEQ ID NO. 1:</u>
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc. Model Number 380A

[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO. 1: from 1269 through 1252 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO: 1 (anti-sense):
(nucleotide sequence of antisense oligo EC-1C)
5' C T G T T G G A A A T C C T A G A A 3' 1252

Site: 1269 through 1252 of the human mRNA for c-raf-1 oncogene

[2] INFORMATION FOR SEQ ID NO. 2:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.2: from 1626 through 1609 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:2:

(nucleotide sequence of antisense oligo EC-2C):
5' T C T T G G T G A G G T C G C A C T 3' 1609

Site: 1626 through 1609 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 3:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.3: from 1857 through 1840 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:3:

(nucleotide sequence of antisense oligo EC-3C):
5' C A A G A A T A T A T C G A A T G A 3' 1840

Site: 1857 through 1840 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 4:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.4: from 89 through 72 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:4:

(nucleotide sequence of antisense oligo DK-1)
5' G G C G G G G T T G C A G G A C A G 3' 72

Site: 89 through 72 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 5:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 17 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.5: from 175 through 158 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:5:

(nucleotide sequence of antisense oligo DK-2)
5' G T T T T G G T A A C G A C T A G 3' 158

Site: 175 TO 158 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 6:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 13 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.6: from 203 through 191 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:6:

(nucleotide sequence of antisense oligo DK-3)
5′ C T C G G T A G T T T G T 3′ 191

Site: 203 through 191 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 7:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.7: from 272 through 255 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:7:

(nucleotide sequence of antisense oligo DK-4)
5′ T C A A A C G G T A G T A G A C T A 3′ 255

Site: 272 through 255 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 8:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 50 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)

[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.8: from 393 through 344 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:8:

(nucleotide sequence of antisense oligo DK-5)

```
5' G T G G A A C T C A   C G A A A G T A T T     374
   C C G T C A G T A C   G T T C G A G T A A     354
   G G T A A A G C G T  3'                       344
```

Site: 393 TO 341 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 9:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 13 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.9: from 488 through 476 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:9:

(nucleotide sequence of antisense oligo DK-6)

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5' C T G C G T C G T A   G T C  3'              476
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Site: 488 through 476 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 10:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear

[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.10: from 660 through 643 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:10:

(nucleotide sequence of antisense oligo DK-7)
5' C G A T G T C A C G A G T A C T T T 3' 643

Site: 660 through 643 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 11:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 20 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.11: from 970 through 951 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:11:

(nucleotide sequence of antisense oligo DK-8)
5' A A C G T A G G A G T T A G T A G G A C 3' 951

Site: 970 through 951 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 12:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 20 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes

[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.12: from 1179 through 1159 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:12:

(nucleotide sequence of antisense oligo DK-9)
5' G T A G T G A A G T G A C C G A A G A T 3' 1159

Site: 1179 through 1159 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 13:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 15 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.13: from 1235 through 1221 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:13:

(nucleotide sequence of antisense oligo DK-10)
5' A C G G T A A A T G G G A A T 3' 1221

Site: 1235 through 1221 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 14:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer

[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.14: from 1313 through 1296 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:14:

(nucleotide sequence of antisense oligo DK-11)
5' C G G T G G A G T A A G G A C T T C 3' 1296

Site: 1313 through 1296 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 15:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.15: from 1373 through 1354 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:15:

(nucleotide sequence of antisense oligo DK-12)
5' A G G A A A A C A G T A C A T G G G G T 1355
A 3' 1354

Site: 1373 through 1354 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 16:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 8 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.16: from 1457 through 1449 of the mRNA of the human c-raf-1 genome in "EUGENE"

[xi] SEQUENCE DESCRIPTION: SEQ ID NO:16:

(nucleotide sequence of antisense oligo DK-13)
5′ T T G T A G A C 3′ 1449

Site: 1457 through 1449 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 17:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 24 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.17: from 1510 through 1487 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:17:

(nucleotide sequence for antisense oligo DK-14)
5′ G T A C G T T T A T C A G G T A A G G G 1491
A C T C 3′ 1487

Site: 1510 through 1487 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 18:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 20 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.18: from 1760 through 1741 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:18:

(nucleotide sequence for antisense oligo DK-15)

5′ G G G C A G T A G T   C A A G T A T G T T 3′   1741

Site: 1760 through 1741 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 19:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 50 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.19: from 1887 through 1838 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:19:

(nucleotide sequence for antisense oligo DK-16)

5′ T C G A T G G T C G   G A G A A G T A A C   1868
G A A A C C C C G T   C A A G A A T A T A   1848
T C G A A T G A T T 3′   1838

Site: 1887 through 1838 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 20:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 39 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.20: from 1931 through 1893 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:20:

(nucleotide sequence for antisense oligo DK-17)

5′ A C C C C T T T T T   C T C C G G A G A G   1912
A A G G A A A T G A   A A G A A G T G T 3′   1893

Site: 1931 through 1893 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 21:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 39 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.21: from 2149 through 2126 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:21:

(nucleotide sequence for antisense oligo DK-18)
5′ T T T C C C T C G T C T T T T C A C C A 2130
C G G A 3′ 2126

Site: 2149 through 2126 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 22:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.22: from 2278 through 2261 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:22:

(nucleotide sequence for antisense oligo DK-19)
5′ G A C G T T T A C C G A A G G A A G 3′ 2261

Site: 2278 through 2261 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 23:
  [i] SEQUENCE CHARACTERISTICS:
    [A] LENGTH: 20 nucleotide bases
    [B] TYPE: deoxyribonucleic acid (DNA)
    [C] STRANDEDNESS: single, anti-sense
    [D] TOPOLOGY: linear
  [ii] MOLECULE TYPE: cDNA to mRNA
  [iii] HYPOTHETICAL: no
  [iv] ANTI-SENSE: yes
  [vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
  [vii] IMMEDIATE SOURCE:
    [A] LIBRARY: "EUGENE" gene library, accessed by computer
  [x] PUBLICATION INFORMATION: none
    [K] RELEVANT RESIDUES In SEQ ID NO.23: from 2425 through 2406 of the mRNA of the human c-raf-1 genome in "EUGENE"
  [xi] SEQUENCE DESCRIPTION: SEQ ID NO:23:

(nucleotide sequence for antisense oligo DK-20)

5' G A T G G A A T G A   A G G A G A T T T A 3'   2406

Site: 2425 through 2406 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 24:
  [i] SEQUENCE CHARACTERISTICS:
    [A] LENGTH: 26 nucleotide bases
    [B] TYPE: deoxyribonucleic acid (DNA)
    [C] STRANDEDNESS: single, anti-sense
    [D] TOPOLOGY: linear
  [ii] MOLECULE TYPE: cDNA to mRNA
  [iii] HYPOTHETICAL: no
  [iv] ANTI-SENSE: yes
  [vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
  [vii] IMMEDIATE SOURCE:
    [A] LIBRARY: "EUGENE" gene library, accessed by computer
  [x] PUBLICATION INFORMATION: none
    [K] RELEVANT RESIDUES In SEQ ID NO.24: from 2626 through 2601 of the mRNA of the human c-raf-1 genome in "EUGENE"
  [xi] SEQUENCE DESCRIPTION: SEQ ID NO:24:

(nucleotide sequence for antisense oligo DK-21)

5' G G G G A T T C T T   T T C A A G G T A T   2607
C A T G G T 3'   2601

Site: 2626 through 2601 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 25:
  [i] SEQUENCE CHARACTERISTICS:
    [A] LENGTH: 26 nucleotide bases

[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.25: from 2678 through 2666 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:25:

(nucleotide sequence for antisense oligo DK-22)
5' T T T T G G T A G G G T T 3' 2666

Site: 2678 through 2666 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 26:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 21 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.26: from 2819 through 2798 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:26:

(nucleotide sequence for antisense oligo DK-23)
5' C A A A C A A A C A A A C A A A C A A T 2799
C 3' 2798

Site: 2819 through 2798 of the human mRNA for c-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 27:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 8 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense

[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.27: from 106 through 99 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:27:

(nucleotide sequence for antisense oligo DK-24)
5′ G G T A G A A C 3′ 99

Site: 106 through 99 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 28:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 8 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.28: from 308 through 301 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:28:

(nucleotide sequence for antisense oligo DK-25)
5′ C G G T A G G G 3′ 301

Site: 308 through 301 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 29:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 23 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes

[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.29: from 950 through 928 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:29:

(nucleotide sequence for antisense oligo DK-26)
5' A G G T A G T G A T   G G A G G A G A T G   931
A T G 3'   928

Site: 950 to 928 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 30:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.30: from 1190 through 1173 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:30:

(nucleotide sequence for antisense oligo DK-27)
5' T A G C G G T A C G   G T G G C G G G 3'   1173

Site: 1190 through 1173 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 31:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:

[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.31: from 1409 through 1392 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:31:

(nucleotide sequence for antisense oligo DK-28)
5' G A C C T G G T A C A G C T T C G C 3' 1392

Site: 1409 through 1392 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 32:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 21 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.32: from 1454 through 1434 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:32:

(nucleotide sequence for antisense oligo DK-29)
5' C T C C A T C A G G T A C G G G A C C C 1435
G 3' 1434

Site: 1454 through 1434 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 33:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 10 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none

[K] RELEVANT RESIDUES In SEQ ID NO.33: from 1975 through 1966 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:33:

(nucleotide sequence for antisense oligo DK-30)
5' T T G A G T A G C C 3' 1966

Site: 1975 through 1966 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 34:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 13 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.34: from 2110 through 2098 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:34:

(nucleotide sequence for antisense oligo DK-31)
5' C G T C G T A G T C C C G 3' 2098

Site: 2110 through 2098 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 35:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 39 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.35: from 2159 through 2119 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:35:

(nucleotide sequence for antisense oligo DK-32)
5′ G T A C C C C T G G   G G G A G T A G A G   2138
G G T C C C A C C C   C T T A C C C C C   3′   2119

Site: 2159 through 2119 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 36:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 16 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.36: from 2266 through 2251 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:36:

(nucleotide sequence for antisense oligo DK-33)
5′ G G G T T T T A A A   T C T T C A   3′   2251

Site: 2266 through 2251 of the human mRNA for A-raf-1 oncogene.

[2] INFORMATION FOR SEQ ID NO. 37:
[i] SEQUENCE CHARACTERISTICS:
[A] LENGTH: 18 nucleotide bases
[B] TYPE: deoxyribonucleic acid (DNA)
[C] STRANDEDNESS: single, anti-sense
[D] TOPOLOGY: linear
[ii] MOLECULE TYPE: cDNA to mRNA
[iii] HYPOTHETICAL: no
[iv] ANTI-SENSE: yes
[vi] ORIGINAL SOURCE: DNA synthesizer, Applied Biosystems, Inc., Model Number 380A
[vii] IMMEDIATE SOURCE:
[A] LIBRARY: "EUGENE" gene library, accessed by computer
[x] PUBLICATION INFORMATION: none
[K] RELEVANT RESIDUES In SEQ ID NO.37: from 2348 through 2331 of the mRNA of the human c-raf-1 genome in "EUGENE"
[xi] SEQUENCE DESCRIPTION: SEQ ID NO:37:

(nucleotide sequence for antisense oligo DK-34)
5′ C T T C C G T G T A   G T C C G T G T   3′   2331

WHAT IS CLAIMED IS:

1. A method for killing cells expressing an activated *ras* oncogene comprising contacting said cells *in vivo* or *in vitro* with a cytotoxically-effective amount of a heterotypic antisense oligonucleotide, or combination of antisense oligonucleotides, or pharmaceutically-effective analogs thereof, said antisense oligonucleotide having a base sequence complementary to the DNA or transcribed messenger RNA of a *raf* gene in said cells.

2. The method of claim 1 wherein the anti-*raf* antisense oligonucleotide is synthesized to contain a nuclease-resistant backbone linkage structure.

3. The method of claim 2 wherein the nuclease-resistant backbone linkage is a phosphorothioate linkage.

4. The method of claim 2 wherein the nuclease-resistant backbone linkage is a methylphosphonate linkage.

Site: 2348 through 2331 of the human mRNA for A-raf-1 oncogene.

5. The method of claim 1 wherein the heterotypic anti-raf antisense oligonucleotide is contacted with said ras-activated cells in vivo by systemic administration to an individual.

6. A method for treating an individual having cancer cells which express an activated ras oncogene, comprising administering to the individual an effective amount of a preparation containing a therapeutic heterotypic anti-raf antisense oligonucleotide or combination of anti-raf oligonucleotides, or pharmaceutically-effective analogs thereof, sufficient to kill the ras-activated cancer cells present in said individual.

7. The method of claim 6 wherein the oligonucleotide preparation is administered systemically.

8. A method for killing ras-activated cancer cells comprising exposing the ras-activated cancer cells to a cytotoxically-effective amount of a heterotypic antisense oligonucleotide, or combination of antisense oligonucleotides, having a base sequence complementary to all or part of the sequence of the DNA or transcribed messenger RNA from

a raf target gene present in said ras-activated cancer cells.

9. The method of claim 8 wherein the antisense oligonucleotide comprises at least 8 nucleotide bases and contains a base sequence complementary to an initiation coding region sequence in the DNA or transcribed messenger RNA from a raf target gene present in the ras-activated cells of said cancer.

10. The method of claim 8 wherein the antisense oligonucleotide comprises at least 8 nucleotide bases and contains a base sequence complementary to a termination coding region sequence in the DNA or transcribed messenger RNA from a raf target gene present in the ras-activated cells of said cancer.

11. The method of claim 8 wherein the ras-activated cancer cells comprise human cancer cells present simultaneously in one or more organs of a host organism.

12. A heterotypic antisense oligonucleotide complementary to all or any part of the DNA or transcribed messenger RNA of a *raf* gene, said oligonucleotide being characterized by having the ability to kill *ras*-activated cancer cells upon contact with said cancer cells *in vivo* or *in vitro*, and the nuclease-resistant backbone linkage structural variants thereof.

13. The oligonucleotide of claim 12 wherein said nuclease-resistant backbone linkage is a phosphorothioate linkage.

14. The antisense oligonucleotide of claim 12 wherein said nuclease-resistant backbone is a methylphosphonate linkage.

15. The oligonucleotide of claim 12 wherein said oligonucleotide has a base sequence from about 8 to about 50 nucleotide base pairs in length.

16. The oligonucleotide of claim 12 wherein said oligonucleotide has the following nucleotide base sequence identified as SEQ ID NO.1:

5′ C T G T T G G A A A T C C T A G A A 3′.

17. The oligonucleotide of claim 12 wherein the

therapeutic oligonucleotide comprises the following 18 base sequence identified as SEQ ID NO.2:

5′ T C T T G G T G A G G T C G C A C T 3′.

18. The oligonucleotide of claim 12 wherein the therapeutic oligonucleotide comprises the following 18 base sequence identified as SEQ ID NO.3:

5′ C A A G A A T A T A T C G A A T G A 3′.

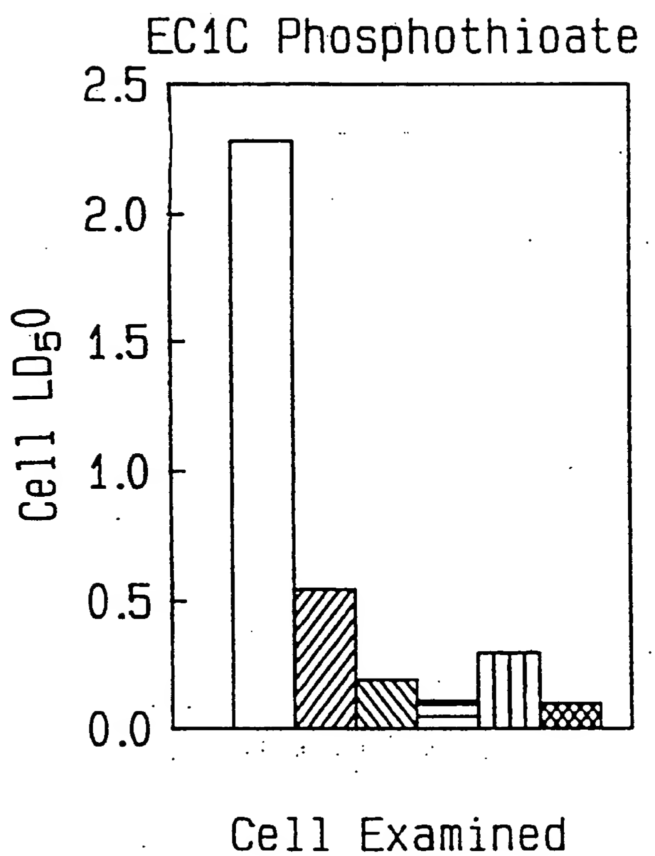
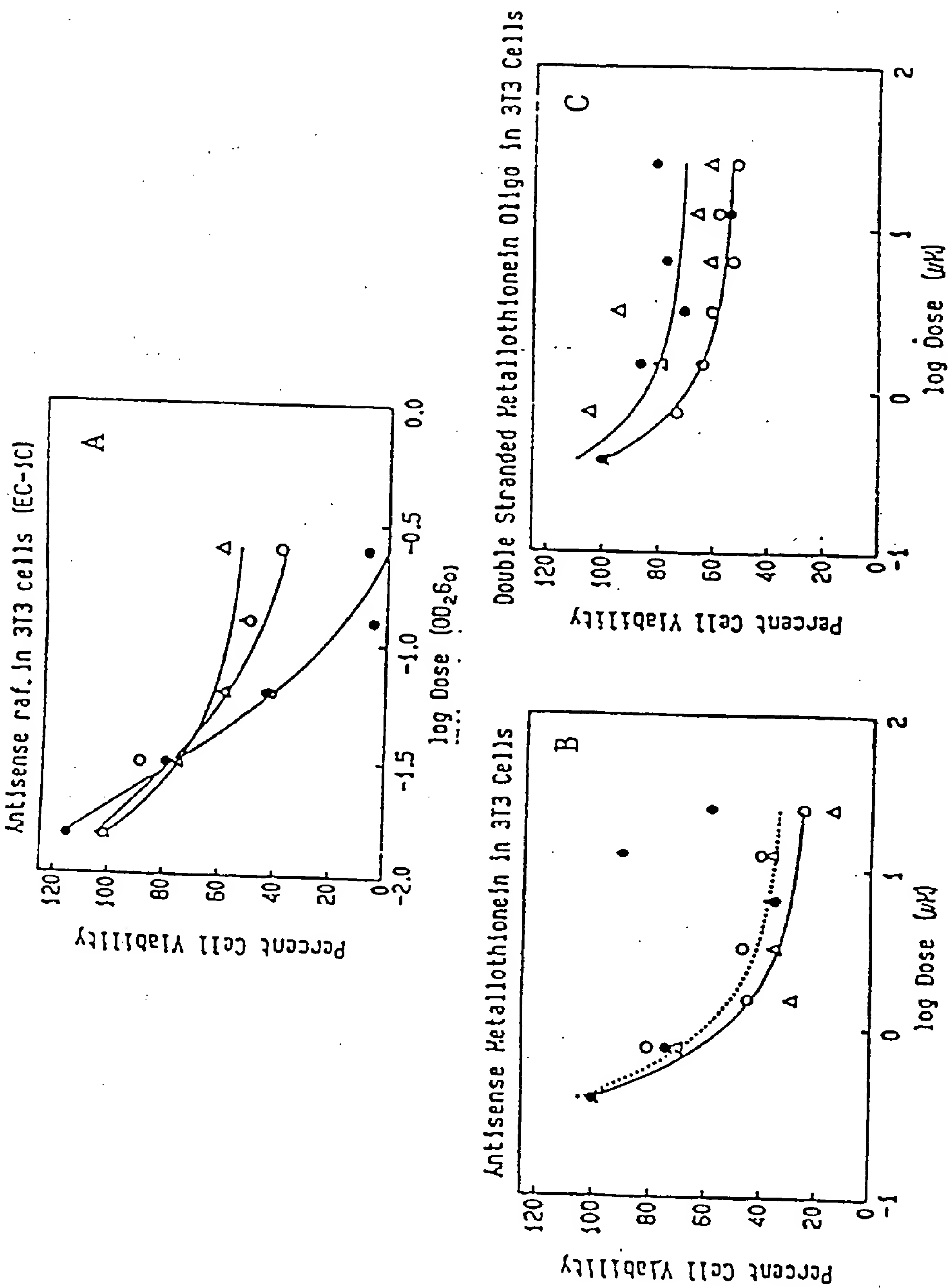

EC1C Phosphothioate
2.5
2.0
1.5
1.0
0.5
0.0
Cell LD_{50}
Cell Examined

FIGURE 1

Figure 2


Antisense raf. in 3T3 cells (EC-1C)
A
Percent Cell Viability
log Dose (OD260)
Antisense Metallothionein in 3T3 Cells
B
Percent Cell Viability
log Dose (μM)
Double Stranded Metallothionein Oligo in 3T3 Cells
C
Percent Cell Viability
log Dose (μM)

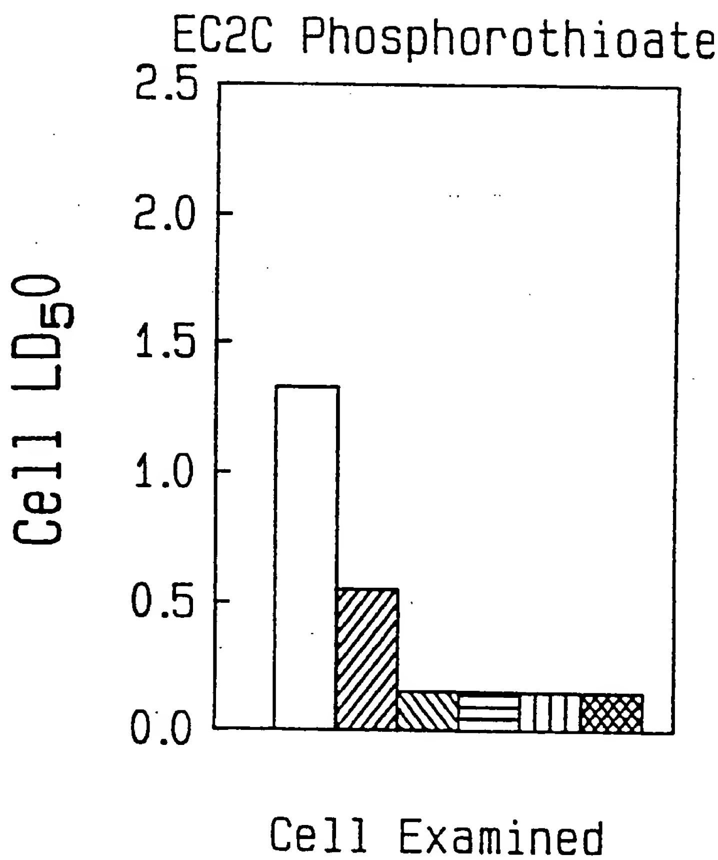

EC2C Phosphorothioate
2.5
2.0
1.5
1.0
0.5
0.0
Cell LD_{50}
Cell Examined

FIGURE 3

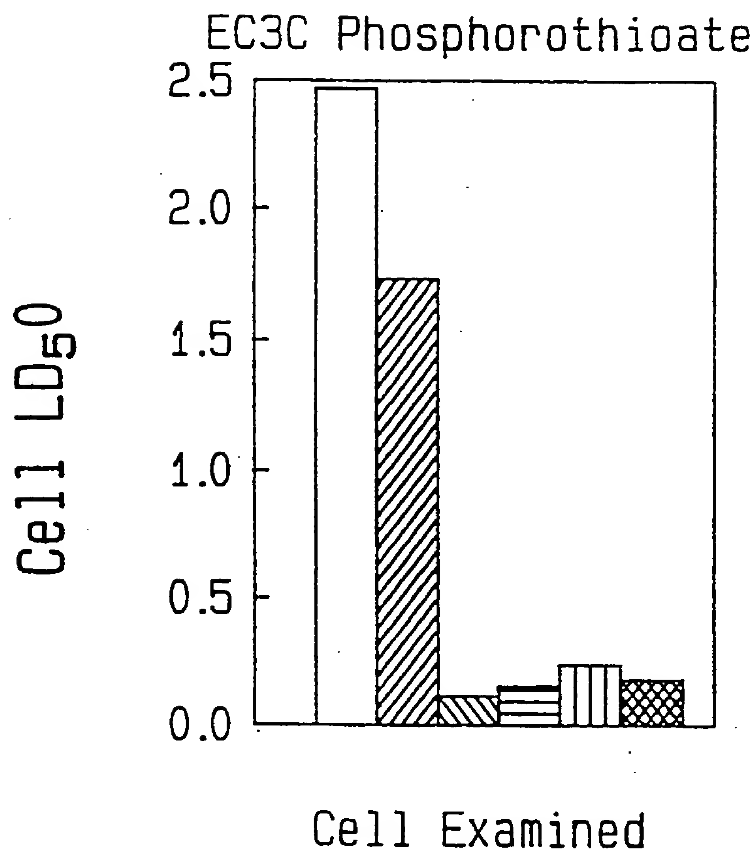

EC3C Phosphorothioate
2.5
2.0
1.5
1.0
0.5
0.0
Cell LD_{50}
Cell Examined

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04091

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00, 31/70, 31/74; C07H 21/04
US CL :514/44; 536/24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Reviews, Volume 90, No. 4, issued June 1990, E. Uhlmann et al., "Antisense Oligonucleotides: A New Therapeutic Principle", pages 543-584, see entire document.	1-18
A, P	Science, Volume 261, issued 20 August 1993, C. A. Stein et al., "Antisense Oligonucleotides as Therapeutic Agents - Is the Bullet Really Magical?", pages 1004-1012, see entire document.	1-18
A, P	Cancer Gene Therapy, Volume 1, No. 1, issued March 1994, B. Y. Tseng et al., "Antisense Oligonucleotide Technology in the Development of Cancer Therapeutics", pages 65-71, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 JULY 1994	JUL 20 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	CHARLES C. P. RORIES, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Cellular Biochemistry, Supplement 16B, issued February 1992, A. Cuadrado, "Functional Link Between Ras and Raf in Oncogenic Transformation", page 245, see entire abstract.	1-18
Y	Nature, Volume 349, issued 31 January 1991, W. Kolch et al., "Raf-1 Protein Kinase is Required for Growth of Induced NIH/3T3 Cells", pages 426-428, see entire document, especially page 428.	1-18
Y	Science, Volume 243, issued 10 March 1989, U. Kasid et al., "Effect of Antisense c-*raf*-1 on Tumorigenicity and Radiation Sensitivity of a Human Squamous Carcinoma", pages 1354-1356, see entire document.	1-18
Y	Journal of Cell Biology, Volume 266, No. 23, issued 15 August 1991, M. Carroll et al., "Erythropoietin Induces Raf-1 Activation and Raf-1 is Required for Erythropoietin-Mediated Proliferation", pages 14964-14969, see entire document, especially page 14968.	1-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04091

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, MEDLINE, Derwent WORLD PATENT INDEX
search terms: raf, ras, antisense, cancer, cdna, gene, nucleotide, sequence, oligonucleotide

DNA sequence search of oligonucleotides having sequences shown as SEQ ID NOs:1-3 in:
EMBL-NEW-4, GenBank 82, GenBank-New 4, UEMBL 38-82, N-GeneSeq 14